Amendments to the Specification:

The following is a marked-up version the Specification pursuant to revised 37 C.F.R. §1.121, with instructions and markings showing changes made herein to the Specification as filed. Underlining denotes added text while strikeout denotes deleted text.

On page 1, after the title, please add the following paragraph:

The present application is a Continuation application of currently pending U.S. Patent Application Serial Number 10/037,677, filed 10/23/01, which is a Divisional of U.S. Patent Application Serial Number 09/314,847, filed May 19, 1999, now U.S. Patent No. 6,365,410.

Please replace the paragraph on page 5, lines 10–17, with the following rewritten paragraph:

The present invention encompasses methods for evolving gram positive and gram negative microorganisms as well as yeast, fungus and eucaryotic cells including hybridomas. In one embodiment, the gram negative microorganism includes members of *Enterobacteriaceae* and in another embodiment comprises *Eschericia* and in another embodiment comprises *E.coli* and *E.blattae*. In further embodiments of the present invention, the evolved microorganism includes ~~E.coli~~ *E.coli* having ATCC accession number PTA-91 and ~~E.blattae~~ *E.blattae* having ATCC accession number PTA-92.

Please replace the paragraph beginning at page 5, line 22, with the following rewritten paragraph:

Figures 1A-1B shows the nucleic acid (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequence of the ~~mutD~~ *mutD* gene. Illustrative examples of mutations of the ~~mutD~~ *mutD* gene are provided.

Please replace the paragraph beginning at page 5, line 24, with the following rewritten paragraph:

Figures 2A-2B provides the nucleic acid sequences (SEQ ID NO:3 and 5, respectively) for the enzyme 1,3-propanediol dehydrogenase (PDD).

Please replace the paragraph beginning at page 5, line 26, with the following rewritten paragraph:

Figures 3A-3B provides the amino acid sequences (SEQ ID NO:4 and 6, respectively) for the enzyme 1,3-propanediol dehydrogenase (PDD).

Please replace the Table on page 6, lines 10-13 and the text immediately following, with the following text:

Depositor Identification Reference	**International Depository Designation**	**Date of Deposit**
Escherichia coli MM294 derivative	ATCC PTA-91	May 1~~7~~9, 1999
Escherichia blattae 33429 derivative	ATCC PTA-92	May 1~~7~~9, 1999

The use of a plasmid comprising a mutator gene, ie, a mutator plasmid, can be used to control the mutation rate of a microorganism. As described under Section II below, plasmid constructs can be designed which provide reduced levels of expression of a mutator gene thereby providing a means for altering the ratio of naturally occurring DNA repair genes vs mutator genes. This provides a means for combining the advantage of mutD mutations (which results in random mutagenesis) with the advantages of the other known mutators (lower mutation frequency which leads to a lower burden on the cells). Additionally, plasmid constructs can be designed that allow for curing the evolved microorganism of the mutator gene, such as the use of a temperature sensitive origin, thereby allowing for a means for turning the mutation events off and on in the microorganism. For a gram positive microorganism, such as *B. subtilis* ~~B.subtilis~~ where the entire genome has been sequenced, the present invention could encompass the steps of deleting or mutating a DNA repair gene, evolving the *Bacillus* ~~Bacillus~~, and restoring the naturally occurring DNA repair system through recombination events. As disclosed herein, several members of ~~Escherichia~~ *Escherichia*, such as ~~E. coli and E. blatte~~ *E. coli* and *E. blattae* have been subjected to the directed evolution methods. Illustrative examples of evolved ~~E.coli and E.blattae~~ *E. coli* and *E. blattae* have been

deposited with the ATCC and have accession numbers ~~and~~ PTA-91 and PTA-92, respectively.

Please replace the paragraph beginning at page 10, line 22, with the following rewritten paragraph:

In illustrative embodiments disclosed herein, a mutated ~~mutD~~ *mutD* gene residing on a plasmid was introduced via recombinant techniques into ~~E.coli or E.blatte~~ *E. coli* or *E. blattae*. The ~~E.coli or E.blatte~~ *E. coli* or *E. blattae* cell was then cultured under conditions suitable for growth for a time sufficient for at least 20 doublings and up to at least about 2000 doublings under conditions of selective pressure. In one example, ~~E.coli~~ *E. coli* was grown under conditions of increased temperature or in the presence of DMF and in another ~~E.blattae~~ *E. blattae* was growth in the presence of solvent, such as DMF or 1,3 propanediol. As a result, ~~E.coli~~ *E. coli* was evolved into a microorganism capable of growing at temperatures up to about 48° C or in the presence of 80g/l DMF. ~~E.coli~~ *E. coli* evolved to grow at elevated temperatures also became auxotrophic for three amino acids, Cys/Met, Asp/Asn and Pro. ~~E.blattae~~ *E. blattae* was evolved into a microorganism capable of growing anaerobically in the presence of at least 105 g/l 1,3-propanediol and which comprised genetic changes in at least one catalytic activity associated with 1,3 propanediol production, 1,3-propanediol dehydrogenase, shown in Figure 3 (SEQ ID NO:4).

Please replace the paragraph beginning at page 12, line 9, with the following rewritten paragraph:

Mutator genes of the present invention include but are not limited to, mutations of the DNA repair genes ~~mutD, mutT, mutY, mutM, mutH, mutL, mutS or mutU~~ *mutD, mutT, mutY, mutM, mutH, mutL, mutS* or *mutU* or their homologues in other microorganisms. A description of the DNA repair genes are disclosed in Miller, *supra*; ~~mutD~~ *mutD* is disclosed in Maki et al., 1983, Proc. Natl. Acad. Sci., U.S.A. 80, 7137-7141 (GenBank accession number K00985.1 GI: 147678 and Figure 1); ~~B. subtilis mutS and mutL~~ *B. subtilis mutS* and *mutL* are disclosed in Ginetti et al., 1996, Microbiology, Aug, 142 (Pt 8): 2021-9; ~~Streptococcus pneumoniae~~ *Streptococcus pneumoniae* hex B repair gene, ~~mutL of Salmonella typhimurium and PMS1 of~~

~~Saccharomyces cerevisiae~~ *mutL* of *Salmonella typhimurium* and PMS1 of *Saccharomyces cerevisiae* are disclosed in Prudhomme et al., 1989, J. Bacteriology, Oct; 171 (10): 5332-8; ~~Streptococcus pneumoniae hexA and mutS of Salmonella typhimurium and E.coli~~ *Streptococcus pneumoniae* hexA and mutS of *Salmonella typhimurium* and *E.coli* are disclosed in Priebe et al., J. Bacteriol, 1988, Jan; 170(1): 190-6 and Prudhomme et al., 1991, J. Bacteriol. Nov; 173(22): 7196-203; human ~~mutS~~ *mutS* homologue, ~~hMSH2~~ *hMSH2*, and human ~~MutL~~ *MutL* homologue, ~~hMLH1~~ *hMLH1*, are disclosed in Macdonald et al., 1998, Heptology, Jul 28(1):90-7; ~~the mut-1 of Neurospora~~ *mut-1* of *Neurospora* is disclosed in Dillon et al.,1994, Genetics, Sep 138(1):61-74 and yeast homologues of ~~mutL and mutS~~ *mutL* and *mutS* are disclosed in WO 97/15657. The methods of the present invention comprise~~s~~ the use of at least one of the mutant DNA repair genes and may involve the use of more than one. It is preferred that a mutator gene be dominant to the wild type gene of the microorganism such that mutations are introduced into the genome of the microorganism. In a preferred embodiment, the mutator gene is a mutation of the ~~mutD~~ *mutD* gene. The nucleic acid and amino acid sequence for mutD is shown in Figure 1 (SEQ ID NO:1 and 2, respectively). One particular ~~mutD~~ *mutD* mutation, ~~mutD5~~ *mutD5*, is disclosed in Takano, K., et al., (1986, *Mol Gen Genet* 205, 9-13, Structure and function of ~~dnaQ and mutD~~ *dnaQ* and *mutD* mutators of Escherichia coli). Strain CSH116 was obtained as disclosed in Miller, J. H. (1992, A Short Course in Bacterial Genetics). This strain is reported to carry the ~~mutD5~~ *mutD5* allele. The ~~mutD~~ *mutD* gene in this strain was found to be very different from the published ~~mutD5~~ *mutD5*. The ~~mutD~~ *mutD* gene from strain CSH116 is designated herein as ~~mutD5~~' *mutD5*'. Table I gives the mutations found in ~~mutD5 and mutD5~~'. *mutD5 and mutD5'.* Further mutations in ~~mutD~~ *mutD* which result in increased levels of mutation frequency were identified recently in Taft-Benz, S. A. et al., (1998, *Nucl. Acids Res.* 26, 4005-4011, Mutational analysis of the 3'-5' proofreading exonuclease of Escherichia coli DNA polymerase III). Table I describes various ~~mutD~~ *mutD* mutations useful in the present invention. Table II describes various promoters used with the ~~mutD~~ *mutD* mutations and Table III describes mutator plasmids and the range of available mutation frequencies in ~~E.coli~~ *E. coli*.

Please replace the Table on page 14, with the following rewritten Table:

Name	Mutations
wild type	ATGACCGCTATG...(SEQ ID NO:7)
pOS100	TTGA-CGCTTTG...(SEQ ID NO:8)
pOS101	**G**TGACCGCT**G**TG...(SEQ ID NO:9)
pOS102	**G**TG-CCGCT**G**TG...(SEQ ID NO:10)
pOS104	TTGACCGCTTTG...(SEQ ID NO:11)
pOS105	**G**TGACCGCT**G**TGAGCACTT**(G)**CAATTACACGCCAGATCGTTCTCGATACCGAA A***T*****(C)**...(SEQ ID NO:12)
pOS106	**G**TGACCGCT-TG...(SEQ ID NO:13)

Please replace the paragraph beginning at page 17, line 17, with the following rewritten paragraph:

Nucleic acid encoding a mutator gene can be isolated from a naturally occurring source or chemically synthesized as can nucleic acid encoding a protein or enzyme. Sources for obtaining nucleic acid encoding DNA repair ~~genes mutD, mutT, mutY, mutM, mutH, mutL, mutS or mutU~~ *mutD, mutT, mutY, mutM, mutH, mutL, mutS* or *mutU* is provided in Section II. Figure 1 provides the nucleic acid (SEQ ID NO:1) and amino acid sequence (SEQ ID NO:2) for mutD and Table I and III provide preferred mutations for ~~mutD~~ *mutD* and the mutation rates obtained for each construct. Once nucleic acid encoding a mutator gene is obtained, plasmids or other expression vectors comprising the mutator gene may be constructed using techniques well known in the art. Molecular biology techniques are disclosed in Sambrook et al., Molecular Biology Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989) and Brown, T. Current Protocols in Molecular Biology, Supplements 21, 24, 26 and 29. Nucleic acid encoding a mutator gene is obtained and transformed into a host cell using appropriate vectors. A variety of vectors and transformation and expression cassettes suitable for the cloning, transformation and expression in bacteria are known by those of skill in the art.

Please replace the paragraph beginning at page 19, line 29, with the following rewritten paragraph:

mutD and *mutD5*' genes were amplified by PCR using mutd1 (5'-CGCCTCCAGCGCGACAATAGCGGCCATC-3' SEQ ID NO:14) and mutd2 (5'-CCGACTGAACTACCGCTCCGCGTTGTG-3' SEQ ID NO:15) primers from genomic DNA *of E. coli* and *E. coli* CSH116 (Miller 1992), respectively. The PCR products were cloned into pCR-Blunt vector (Invitrogen, Carlsbad, CA). Plasmids from clones with the correct orientation were isolated and digested with *Sma*I-*Hind*III restriction enzymes. The overhang ends were filled using T4 polymerase and cloned into pMAK705 plasmid digested with *Sma*I-*Pvu*II. The ligation products were transformed into JM101 competent cells. The resulted plasmids had the temperature-sensitive origin of replication, carried kanamycin resistance marker and were named pMutD-71 (control plasmid, wild type genotype) and pMutD5-61 (mutator plasmid).

Please replace the paragraph beginning at page 26, lines 5-15, with the following rewritten paragraph:

The evolution of 1,3-propanediol resistance was faster in the presence of B12. After 2 months of evolution GEB025 (+B12) was able to grow with 95-100g/l 1,3-propanediol. After 3 months of anaerobic growth under selection in the presence of 1,3-propanediol, GEB028 (-B12) could grow in medium supplemented with 110g/l 1,3-propanediol. Analysis of aerobic growth of GEB031 on LB plates supplemented with 85, 95, 105 and 115g/l 1.3-propanediol showed that cells produce much bigger colonies in the presence of 85g/l in comparison with 105g/l. No growth was observed at 115g/l 1,3 propanediol. The results indicate that after 3 months of applying directed evolutions techniques described herein to *E.blattae*, the tolerance to 1,3 propanediol was increased from 75 g/l to at least 105 g/l under aerobic conditions. The plasmid was cured from the GEB031 strain by growing at 41.5 degrees. An illustrative clone, GEB031-4 was deposited with the ATCC and has accession number PTA-92.

Please replace the paragraph beginning at page 26, lines 25-26, with the following rewritten paragraph:

Strains - Wild type ATCC 33429, *E.blattae* ~~E.blattae~~ comprising the mutant PDD as described in Example 4 and having ATCC accession number PTA-92.

Please replace the description of Examples 6 and 6, beginning on page 27, line 30, through page 28, line 35, with the following rewritten description:

Example 6: Cloning and sequencing the 1,3-propanediol dehydrogenase genes (*dhaT)* from *E. blattae*.

The *dhaT* genes were amplified by PCR from genomic DNA from *E. blattae* as template DNA using synthetic primers (primer 1 and primer 2) based on the *K. pneumoniae dhaT* sequence and incorporating an ~~XbaI~~ *Xba*I site at the 5' end and a ~~BamHI~~ *Bam*HI site at the 3' end. The product was subcloned into pCR-Blunt II-TOPO (Invitrogen). The ~~cloning~~ cloned dha*T* ~~were~~ was then sequenced ~~was~~ with standard techniques.

The results of the DNA sequencing are given in SEQ ID NO:~~1~~3 and SEQ ID NO:~~2~~ 4.

Primer 1
5' TCTGATACGGGATCCTCAGAATGCCTGGCGGAAAAT3 ' SEQ ID NO:14

Primer 2
5' GCGCCGTCTAGAATTATGAGCTATCGTATGTTTGATTATCTG3' SEQ ID NO:15

As will be readily understood by the skilled artisan, nucleic acid sequence generated via PCR methods may comprise inadvertent errors. The present invention also encompasses nucleic acid encoding PDD obtainable from ~~E.blattae~~ *E. blattae* having ATCC accession number ——— PTA-92.

Example 7: Comparison of wild-type ~~E.blattae~~ *E.blattae* (ATCC accession number 33429) and the evolved strain GEB031-4 (ATCC accession number ——— PTA-92).

This example shows that *E.blattae* subjected to the methods of the present invention and having ATCC accession number ——— PTA-92 can completely consume 800mM glycerol during anaerobic fermentation and does not accumulate 3-hydroxy-propionaldehyde (3HPA) and does not lose viability. In contrast, the wild-type ~~E.blattae~~ *E.blattae* accumulates 50mM 3 HPA and becomes non viable after consuming only 350 mM glycerol.

The wild-type ~~E.blattae~~ *E.blattae* and the evolved ~~E.blattae~~ *E.blattae* were subjected to fermentation in the following medium: 75 g glycerol, 5 g $K_2HPO_4 \cdot 3H_2O$, 3 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, 4 mg $CoCl_2 \cdot 2H_2O$, 2 g yeast extract, and 1 g peptone per liter water. The pH was maintained with 20% NaOH. Both fermentations were run at 30°C with a N_2 sparge and were inoculated with a stationary grown overnight preculture.